The easy way of analysing vitamins – with innovative bioanalytical methods

Food products are now being enriched and fortified with vitamins in many forms. Food manufacturers, regulatory agencies and commercial laboratories should therefore have analytical methods on hand that allow them to quickly and reliably determine the natural and added vitamin content of food products.

The established microbiological methods for analysis of water-soluble vitamins, which have been on the market for several decades, are both highly sensitive and highly specific. Complementary to microbiological procedures, HPLC, LC/MS/MS and other chromatographic methods are also available. HPLC with UV detection is the method of choice for the analysis of fat-soluble vitamins.

The principles

The principles of microbiological vitamin analysis can be described as follows: certain microorganisms replicate only in the presence of the specific vitamin. These microorganisms must first be cultured in an optimal culture medium before testing. When inoculated on a culture medium that does not contain the specific vitamin, the organisms do not grow. When a standard or sample, containing the missing vitamin, is added, the organisms begin to grow. Growth of the organisms is reflected by turbidity of the culture medium. The degree of turbidity can be measured using a microtiter plate photometer.

Traditional microbiology

In traditional microbiology, colonies of the target microorganisms must first be cultured and later maintained by regular inoculation. Before the actual assay procedure can begin, the cultures must be freshly prepared, and the number of microorganisms must be regulated before the organisms are transferred to the medium. This requires a great deal of time and manpower. Studies of accuracy and precision data have shown that these methods often do not produce satisfactory results.

Figure 1: VitaFast® Folic Acid test kit

The ifp-Institute of Product Quality, in Berlin, has developed a series of microbiological vitamin analysis products with a ready-to-use microtiter plate format. This product line is marketed by R-Biopharm AG (Darmstadt) under the trade name VitaFast® (Figure 1).

Vita®Fast microbiological microtiter plate test to determine watersoluble B-Vitamins

The wells of the microtiter plate are coated with specific microorganisms that metabolise the target vitamin. The technically demanding work of preparing and maintaining microorganism cultures and suspensions is not necessary. The number of microorganisms in the wells is set and optimised in accordance with the respective target vitamin. For test preparation, it is only necessary to add the assay-medium, incremental concentrations of the target vitamin and sample extracts to the wells. There are no washing steps necessary. Once this is done, the microtiter plates are incubated and vitamin measurements and analyses are subsequently performed using a microtiter plate photometer. In addition to assay-medium and sterile water, the test kit contains a characterised standard, which must be serially diluted in simple dilution steps.

The procedure for vitamin B12 analysis with the VitaFast® Vitamin B12 test kit is described in the flow chart over the page.

SAMPLE EXTRACTION

- Add 1g (ml) of sample to a 50ml centrifuge tube
- Add 20ml deionised water, then add 250µl NaCN solution (1%, freshly prepared) and shake; adjust pH to 4.5
- Alternative: Acetate buffer (pH 4.5) can be used instead of deionised water
- Add 300mg taka diastase, shake and incubate for 1 hour at 37°C in the dark; shake occasionally. Fill to 40 ml mark with deionised water and heat in a 95°C water bath for 30 minutes. Afterwards, chill down quickly below 30°C
- Transfer 1ml of extracted sample to a sterile 1.5 ml reaction vial and centrifuge for 5 minutes (> 8000g)

ASSAY MEDIUM

- Remove the desiccant using tweezers
- Add 10ml sterilised water from the test kit
- Heat at 95°C for 5 minutes and chill down quickly below 30°C
- Filter through sterile filter (0.2µm) into a sterile 15ml centrifuge vial

SAMPLE DILUTION

- Perform calculations for dilution of sample extract
- Fill microtiter plate manager
- Dilute clear supernatant

STANDARD CURVE

- Reconstitute standard from the test kit

Standard curve in µg / 100 g (ml)	Sterile water in µl		Standard concentrate in µl		Total volume in µl
Blank: 0	900	+	0	=	900
Standard 1: 0.03	900	+	100	=	1000
Standard 2: 0.06	400	+	100	=	500
Standard 3: 0.09	350	+	150	=	500
Standard 4: 0.12	300	+	200	=	500
Standard 5: 0.18	200	+	300	=	500

MICROTITER PLATE

- Place the required amount of strips in the additional strip holder
- Return the remaining strips with the desiccant to the foil bag
- Pipette 150µl assay-medium to each well
- Pipette 150µl standard or diluted sample extract per well
- Cover strips with adhesive foil
- Incubate in the dark at 37°C for 44–48 hours

MEASUREMENT AND ANALYSIS

- Press adhesive foil to secure
- Turn microtiter plate upside down, shake and invert to the regular position
- Destroy any air bubbles if necessary
- Measure the turbidity in microtiter plate photometer and measure at 610–630nm or at 540–550nm (alternatively)

VitaFast® test kits have been investigated and validated with reference materials in numerous comparison studies. The products were also tested in official interlaboratory ring trials. A summary of some of the data is shown in Table 1. Examples of further validation parameters are presented in Tables 2 and 3.

Table 1: Results for vitamin analysis of reference materials

Test material		Folic acid µg/100g	Vitamin B12 µg/100g	Biotin µg/100g	Niacin mg/100g	Pantho-thenic acid mg/100g	Vitamin B1 mg/100g	Vitamin B2 mg/100g	Vitamin B6 mg/100g
NIST 1846 Baby food	Target	129 (101-157)	3.9 (3.6-4.2)	41.1 (34.5-47.7)	6.33 (5.6-7.1)	4.87 (4.1-5.6)	0.86 (0.7-1.0)	1.74 (1.6–1.8)	0.69 (0.6-0.8)
	VitaFast®	**133**	**4.0**	**40.3**	**6.27**	**4.63**	**0.82**	**1.74**	**0.68**
AACC VMA 399 Cereals	Target	1395 (1160-1620)	21.2 (12.2-25.0)		74.96 (66.7-82.2)	37.35 (31.0-41.9)	5.45 (4.8-6.5)	5.97 (4.9-7.6)	6.99 (6.0-8.3)
	VitaFast®	**1363**	**20.8**		**74.84**	**38.23**	**5.42**	**5.79**	**6.90**
BCR CRM 121 Flour	Target	50 (43-57)					0.36 (0.33-0.4)		
	VitaFast®	**48.5**					**0.36**		
BCR CRM 421 Milk powder	Target	142 (128-156)	3.4 (2.9-3.9)		6.8 (6.6-7.0)		0.51 (0.47-0.55)	1.45 (1.4-1.5)	0.55 (0.5-0.6)
	VitaFast®	**136**	**3.2**		**6.7**		**0.50**	**1.44**	**0.57**
FAPAS® T2130 Baby food	Target		1.46 (0.82-2.11)						
	VitaFast®		**1.69**						
FAPAS® 2133 Liquid vitamins	Target						6.60 (5.5-7.7)		7.71 (6.5-9.0)
	VitaFast®						**7.29**		**7.60**
FAPAS® 2139 Liquid concentrate	Target						8.12 (6.8-9.5)	8.86 (5.3-12.4)	9.02 (7.6-10.5)
	VitaFast®						**8.60**	**8.43**	**9.50**
FAPAS® 2141 Breakfast cereals	Target	458 (342-575)			21.3 (18.2-24.3)			2.07 (1.7-2.5)	2.07 (1.7-2.5)
	VitaFast®	**509**			**21.6**			**2.07**	**2.01**
FAPAS® 2143 Baby food powder	Target		1.73 (0.97-2.50)						
	VitaFast®		**1.76**						
FAPAS® 2148 Breakfast cereals	Target							1.99 (1.6-2.4)	2.05 (1.6-2.5)
	VitaFast®							**2.17**	**2.08**
FAPAS® 2150 Baby food powder	Target		1.60 (0.9-2.3)						
	VitaFast®		**1.58**						

Table 2: Coefficient of variation of VitaFast® Folic Acid standard curves

	Blank	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
Absorption (n=6)	0.066	0.147	0.321	0.694	0.944	1.087
Coefficient of variation (CV in %)	1.6	2.1	1.5	1.7	0.6	1.3

The VitaFast® microtiter plate system has excellent handling and performance characteristics. Unlike other immunological assay systems, no washing steps are required. The test excels through high accuracy and precision. The coefficient of variation (CV) is less than 10%. In analyses of real matrices, the recovery rates ranged from 95 to 105%. The high level of accuracy can be attributed to the test format. All test steps and test reagents (standard, assay-medium, microorganisms) are optimally adjusted and in harmony with each other. Samples with an added or natural vitamin content can be analysed. The sample preparation varies accordingly. With added vitamin samples, a single hot extraction step is generally sufficient for vitamin extraction.

Table 3: Inter-assay batch-to-batch conformity of VitaFast® Folic Acid results with target concentration of reference material AACC VMA 399 (Cereals)

Batch	AACC VMA 399 Target concentration in µg / 100 g	VitaFast® measurement µg folic acid / 100 g
QSKF 39479	1395 (1160 – 1620)	1299
QSKF 39423		1330
QSKF 39374		1322
QSKF 39335		1359
QSKF 39280		1374
QSKF 39129		1423
QSKF 39045		1275
QSKF 38835		1319
QSKF 38812		1468
QSKF 38791		1391
Mean		1356
Standard deviation		59.3
Coefficient of Variation (%)		4.4

Table 4: Folic acid content of different samples determined using EASI-EXTRACT® FOLIC ACID

Sample	Target concentration (in µg / 100 g)	EASI-EXTRACT® FOLIC ACID result	Recovery rate (%) (EASI-EXTRACT® FOLIC ACID result / target conc. x 100)
Premix	85500	86342.5	101%
Baby food, powdered	64.9	101.7	157%
Baby food	100	127	127%
FAPAS® Breakfast cereals	438	458	104.5%
Dietary milk powder	60	58.9	98%
Corn flakes	166	229.7	138%
Cereals	24	20	84%
Soy milk	14	19.4	139%
NIST 1846 Baby food	101-157	108.5	84%

Extraction of natural vitamins should be performed by hydrolysis (enzyme digestion).

The microtiter plate format permits a high degree of automation, and the investment costs for automation are relatively low. Compared to traditional microbiological vitamin assays, the time required for VitaFast® assay performance is roughly 60–70 % less, and materials consumption is around 30 times lower. The assay was developed for analysis of all water-soluble vitamins and substances with analogue effects. Selected amino acids (lysine, methionine and cystine) are also available.

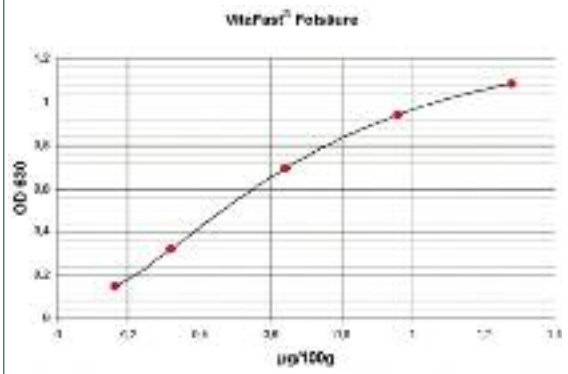
Note: Food products are generally overdosed with vitamins. Test results of over 100% of the target concentration are therefore to be expected.

Immunoaffinity Columns

Apart from the VitaFast® microtiter plate systems for vitamin analysis, which have now been described in detail, R-Biopharm also supplies immunoaffinity column (IAC) systems for preparation of samples for HPLC analysis. These columns are marketed under the trade name EASI-EXTRACT®. In addition to the Vitamin B12 IAC, a new IAC kit for folic acid was recently introduced. Conventional HPLC methods for analysis of folic acid in complex food products have proved to be difficult because the concentrations of folic acid are often very low. Moreover, pigments and interfering substances present in the samples can mask folic acid on HPLC chromatograms. EASI-EXTRACT® FOLIC ACID immunoaffinity columns solve these problems: These systems use highly specific monoclonal antibodies to extract and concentrate the folic acid in the samples and simultaneously wash pigments and interfering components out of the column. When EASI-EXTRACT® FOLIC

A representative standard curve for VitaFast® Folic Acid is shown in figure 2 below.

Figure 2: Standard Curve



ACID is used, the samples extracted for HPLC analysis are purer and sensitivity is improved. EASI-EXTRACT® FOLIC ACID is a validated method for detection of added folic acid in a number of different food products. The immunoaffinity columns have a detection range of 10 to 100,000 µg folic acid per 100g. The IAC system has been successfully implemented for analysis of vitamin tablets, vitamin premixes, cereals, flour, baby food (powder and milk), dietary milk powder and soy milk. EASI-EXTRACT® FOLIC ACID was also evaluated in tests using reference samples of powdered baby food supplied by the National Institute of Standards and Technology (NIST) and FAPAS® cereal samples. All of the results were within the target range. Table 4 shows the range of different samples tested on EASI-EXTRACT® FOLIC ACID immunoaffinity columns.

R-Biopharm's wide range of analytical methods guarantees its customers individual solutions for a range of different analytical problems

The palette of available vitamin analysis products has been expanded by the enzyme immunoassays that were introduced several years ago, namely, RIDASCREEN®FAST Vitamin B12, FOLIC ACID and RIDASCREEN® Biotin.

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